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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68, C12P 19/34

A1

(11) International Publication Number: WO 94/17206

(43) International Publication Date: 4 August 1994 (04.08.94)

(21) International Application Number: PCT/US93/10883

(22) International Filing Date: 12 November 1993 (12.11.93)

(30) Priority Data:
08/010,433 27 January 1993 (27.01.93) US

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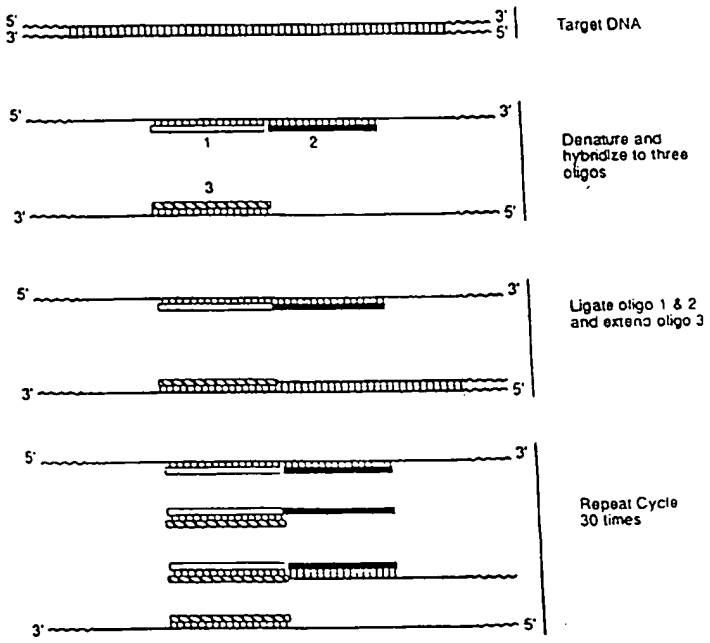
(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHOD FOR AMPLIFYING NUCLEIC ACID SEQUENCES

(57) Abstract

A process for amplifying nucleic acid sequences from a DNA or RNA template which may be purified, or may exist in a mixture of nucleic acids. The resulting nucleic acid sequences may be exact copies of the template, or may be modified. The process has advantages over prior art amplification processes in that it increases the fidelity of copying a specific nucleic acid sequence, and it allows one to more efficiently detect a particular point mutation in a single assay.



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METHODS FOR AMPLIFYING NUCLEIC ACID SEQUENCES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a process for amplifying nucleic acid sequences. More specifically, it relates to an improved process for producing nucleic acid sequences from a DNA or RNA template which may be purified, or may exist in a mixture of nucleic acids. The resulting nucleic acid sequences may be exact copies of the template, or may be modified.

2. Description of Related Art

In the past, methods have been employed for amplifying nucleic acid sequences wherein both strands of the nucleic acid sequence to be amplified are synthesized by the same method. Such methods are prone to limitations due to the nature of the enzymes utilized in these processes.

In U.S. Patents No. 4,683,195 and 4,683,202, DNA or RNA is amplified by the polymerase chain reaction (PCR). These patents are incorporated herein by reference in their entirety. This method involves the hybridization of an oligonucleotide primer to the 5' end of each complementary strand of the double-stranded target nucleic acid. The primers are extended from the 3' end in a 5' → 3' direction by a DNA polymerase which incorporates free nucleotides into a nucleic acid sequence complementary to each strand of the target nucleic acid. After dissociation of the extension products from the target nucleic acid strands, the extension products become target sequences for the next cycle. In order to obtain satisfactory amounts of the amplified DNA, repeated cycles must be carried out, between which cycles, the complementary DNA strands must be denatured under elevated temperatures.

Traditional polymerases used in this process, such as *E. coli* DNA polymerase I have the limitation of being

inactivated at temperatures necessary for the denaturation of the complementary strands. Thus, between each cycle of synthesis by such polymerases and after the heat denaturation step, a fresh aliquot of enzyme must be added to the reaction mixture so that extension of the primer and synthesis of the complementary strand may occur in the following cycle. This additional step increases the time required for amplification and decreases the ease of amplification which requires multiple steps.

In recent years, thermostable DNA polymerases have been discovered and isolated from thermophilic organisms such as *Thermus aquaticus*. Such thermostable polymerases make it possible to add enzyme at the beginning of a series of synthesis and denaturation steps, without the need to add a new aliquot of enzyme after each denaturation step.

A potential problem associated with PCR is the hybridization of a primer sequence to regions of the DNA molecule not intended to be amplified. Generally these undesired hybridizations occur because the target sample contains, in addition to the target sequence itself, other sequences with some complementarity to the primer sequences. If the 3' terminal nucleotides of the primer molecule are successfully hybridized to a sequence other than the target sequence, it is possible that primer extension may be successfully initiated by the polymerase enzyme, leading to the generation of an extension product different from the desired target sequence. Under some circumstances, this extension product will undergo exponential amplification, and be erroneously thought to be the desired target sequence.

A method of detecting a specific nucleic acid sequence present in low copy in a mixture of nucleic acids, called ligase chain reaction (LCR), has also been described. European patent application 0 320 308 describes this method and is incorporated herein by reference in its entirety. Target nucleic acid in a sample is annealed to probes

containing contiguous sequences. Upon hybridization, the probes are ligated to form detectable fused probes complementary to the original target nucleic acid. The fused probes are disassociated from the nucleic acid and serve as a template for further hybridizations and fusions of the probes, thus amplifying geometrically the nucleic acid to be detected. The method does not use DNA polymerase.

LCR has disadvantages due to the need for at least four separate oligonucleotide probes for amplification. It also requires that the entire sequence of the target nucleic acid be known. Further, background signal can be caused by target independent ligation of the probes. Since the third probe hybridizes to the first probe and the fourth probe hybridizes to the second probe, the probes, when added in excess, can easily form duplexes among themselves which can be ligated independently of the target nucleic acid.

European Application No. 0 439 182 which is incorporated herein in its entirety by reference discloses a method of improving LCR amplification by providing probes/primers which hybridize to the target nucleic acid wherein one end is modified such that ligation cannot occur until the modified end is corrected. One such modification is the placement of a small gap between the probes preventing ligation of the probes. The gap sequence of the target nucleic acid must be selected such that the DNA sequence is comprised of three or less different nucleotides from the four possible nucleotides. The fourth nucleotide must be the first base complementary to the 5' end of the adjacent probe. The gap is then filled using a DNA polymerase or reverse transcriptase to extend one or more of the probes in a 5' to 3' direction in a target dependent manner to render the probes ligatable. The reaction mixture omits the fourth deoxynucleoside triphosphate complementary to the base at the 5' end of the adjacent probe. Because this

method requires that the gap chosen in the target nucleic acid only contains bases which are complementary to a maximum of three of the deoxynucleoside triphosphates, the method limits the location of the gap on the target nucleic acid and also limits the size of the gap. Further, the method requires four primers. The application also discloses a method of PCR amplification wherein one end of the primer is modified such that the primer is not extendable by a polymerase enzyme. When this modification is removed in a template dependent manner, the primer can be extended. However, this type of PCR requires an additional step of removal of the modification before extension can occur.

In view of the foregoing disadvantages attendant with prior art methods of amplifying nucleic acid sequences, it should be apparent that there exists a need in the art for a method in which the fidelity of amplified sequences can be increased, which allows for the detection of a particular nucleic acid strand, and which allows one to efficiently examine multiple alleles in a single series of amplification steps.

SUMMARY AND OBJECTS OF THE INVENTION

The present invention is based on the discovery that certain aspects of LCR and PCR can be used in combination to detect and amplify a target nucleic acid sequence with increased fidelity. Accordingly, in one of its process aspects, the present invention relates to a process for amplifying enzymatically a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, comprising the steps of:

a. selecting the target nucleic acid sequence;

b. providing primers, said primers comprising a first primer which is substantially complementary to a first segment at a first end of the target nucleic acid sequence and a second primer which is substantially

complementary to a second segment at a second end of the target nucleic acid sequence and whose 3' end is adjacent to the 5'end of the first primer and a third primer which is similar to the first end of the target nucleic acid sequence and which is substantially complementary to at least a portion of said first primer;

c. providing at least four different nucleotide bases;

d. hybridizing said first and second primers to the target nucleic acid sequence in a target dependent manner to form a primer-target complex;

e. ligating under conditions such that the adjacent 5' end of the first primer and the 3' end of the second primer will ligate to form a fused amplification product substantially complementary to said target nucleic acid sequence;

f. dissociating said fused amplification product from said target nucleic acid sequence;

g. hybridizing said third primer to said fused amplification product;

h. extending said third primer in the presence of the nucleotide bases under conditions such that an extended amplification product is formed substantially complementary to said fused amplification product;

i. dissociating the extended amplification product from the fused amplification product.

In another of its process aspects, the present invention relates to a process for detecting enzymatically a point mutation or allele of a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids using the method disclosed above. One of said primers is comprised of a number of similar oligonucleotide sequences, one of which is exactly complementary to the possible allele or point mutation and each of which oligonucleotides is labeled with a different label. The allele is determined by detecting which labeled oligonucleotide is

contained within the resulting amplification products.

In a third aspect, the present invention relates to a process for amplifying enzymatically a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids comprising the steps of:

a. selecting the target nucleic acid sequence;

b. providing primers, said primers comprising a first primer which is substantially complementary to a first segment at a first end of the target nucleic acid sequence and a second primer which is substantially complementary to a second segment at a second end of the target nucleic acid sequence said second segment being spaced from said first segment and a third primer which is similar to the first end of the target nucleic acid sequence and which is substantially complementary to a portion of said first primer;

c. providing at least four different nucleotide bases;

d. hybridizing said first and second primers to the target nucleic acid sequence in a target dependent manner to form a primer-target complex;

e. extending a 3' end of the second primer in the presence of the nucleotide bases under conditions such that an extended second primer is formed wherein the 3' end of the extended second primer terminates at a base adjacent to a 5' end of the first primer;

f. ligating the ends of the first and second extended primers under conditions such that said first and said extended second primers will form a fused amplification product substantially complementary to said target nucleic acid sequence;

g. dissociating said fused amplification product from said target nucleic acid sequence;

h. hybridizing said third primer to said fused amplification product;

i. extending said third primer in the presence of

the nucleotide bases under conditions such that an extended amplification product is formed substantially complementary to said fused amplification product; and

j. dissociating the extended amplification product from the fused amplification product.

In one of its product aspects, the present invention relates to a kit for amplifying a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids comprising: first, second and third primers; a ligating enzyme; a polymerizing enzyme; and at least four nucleotides.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts one embodiment of the method of DNA amplification/detection as set forth herein.

Figure 2 is a printout from a Phosphor Imager of a scanned acrylamide gel. The arrow indicates the resulting higher molecular weight amplification products.

Figure 3 depicts another embodiment of the method of DNA amplification/detection as set forth herein.

Figure 4 shows the sequence of the multidrug resistance gene (MDR-1)(SEQ ID NO:1).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Prior to discussing this invention in detail, the following terms will first be defined:

The "target nucleic acid" or "target nucleic acid sequence" suitable for use in the present invention may be taken from DNA or RNA, and it may be isolated or present in samples which contain nucleic acid sequences in addition to the target nucleic acid sequence to be amplified. The target nucleic acid sequence may be located on a nucleic acid strand which is longer than the target nucleic acid sequence. In this case, the ends of the target nucleic acid sequence are the boundaries with the unselected nucleic acid sequence and the target nucleic acid sequence.

The target nucleic acid sample may be obtained synthetically, or can be isolated from any organism by methods well known in the art. Particularly useful sources of nucleic acid are derived from tissues or blood samples of an organism, nucleic acids which are present in self-replicating vectors, and nucleic acids derived from viruses and pathogenic organisms such as bacteria and fungi. Also particularly useful for the present invention are target nucleic acid sequences which are related to disease states, such as those caused by chromosomal rearrangement, insertions, deletions, translocations and other mutations, those caused by oncogenes, and those associated with cancer.

The term "selected" means that a target nucleic acid sequence having the desired characteristics is located and probes are constructed around appropriate segments of the target sequence.

The term "probe" or "primer" has the same meaning herein, namely, an oligonucleotide fragment which is single stranded. The term "oligonucleotide" means DNA or RNA.

A probe or primer is "substantially complementary" to the target nucleic acid sequence if it hybridizes to the sequence under renaturation conditions so as to allow target dependent ligation or extension. Renaturation depends on specific base pairing between A-X (where X is T or U) and G-C bases to form a double stranded duplex structure. Therefore, the primer sequence need not reflect the exact sequence of the target nucleic acid sequence. However, if an exact copy of the target nucleic acid is desired, the primer should reflect the exact sequence. Typically, a "substantially complementary" primer will contain at least 70% or more bases which are complementary to the target nucleic segment. More preferably 80% of the bases are complementary and most preferably 90% of the bases are complementary. Generally, the primer must hybridize to the target nucleic acid sequence at the end to

be ligated or extended to allow target dependent ligation or extension.

The primers may be RNA or DNA, and may contain modified nitrogenous bases which are analogs of the normally incorporated bases, or which have been modified by attaching labels or linker arms suitable for attaching labels. Inosine may be used at positions where the target sequence is not known, or where it may be degenerate. The oligonucleotides must be sufficiently long to allow hybridization of the primer to the target nucleic acid and to allow amplification to proceed. They are preferably 15 to 50 nucleotides long, more preferably 20 to 40 nucleotides long, and most preferably 25 to 35 nucleotides long. The nucleotide sequence, content and length will vary depending on the sequence to be amplified.

It is contemplated that a primer may comprise one or more oligonucleotides which comprise substantially complementary sequences to the target nucleic acid sequence. Thus, under less stringent conditions, each of the oligonucleotides would hybridize to the same segment of the target nucleic acid. However, under increasingly stringent hybridization conditions, only that oligonucleotide sequence which is most complementary to the target nucleic acid sequence will hybridize. The stringency of conditions is generally known to those in the art to be dependant on temperature, solvent and other parameters. Perhaps the most easily controlled of these parameters is temperature and since the conditions here are similar to those of PCR, one skilled in the art could determine the appropriate conditions required to achieve the level of stringency desired.

Oligonucleotide primers or oligonucleotide probes suitable for use in the present invention may be derived by any method known in the art, including chemical synthesis, or by cleavage of a larger nucleic acid using non-specific nucleic acid-cleaving chemicals or enzymes, or by using

site-specific restriction endonucleases.

The primers may be prepared using the ß-cyanoethyl-phosphoramidite method or other methods known in the art. A preferable method for synthesizing oligonucleotide primers is conducted using an automated DNA synthesizer by methods known in the art. Once the desired oligonucleotide primer is synthesized, it is cleaved from the solid support on which it was synthesized, and treated, by methods known in the art, to remove any protecting groups present. The oligonucleotide primer may then be purified by any method known in the art, including extraction and gel purification. The concentration and purity of the oligonucleotide primer may be examined on an acrylamide gel, or by measuring the optical densities at 260 and 280 nm in a spectrophotometer.

In order for the ligase to ligate the oligonucleotide primers, the primers used in the present invention are preferably phosphorylated at their 5' ends. This may be achieved by any method known in the art, but is preferably conducted with the enzyme T4 polynucleotide kinase. The oligonucleotides can be phosphorylated in the presence of unlabeled or labeled ATP. In order to monitor the amplification process, labeled ATP may be used to phosphorylate the primers. Particularly preferable is [γ-^{32}P] ATP.

The oligonucleotide primers may alternatively be labeled with any detectable marker known in the art, including other radioactive nuclides such as ^{35}S or ^{3}H and the like, fluorescent markers such as fluorescein, rhodamine, Texas red, Lucifer yellow, AMCA blue and the like, or with enzymatic markers which may produce detectable signals when a particular chemical reaction is conducted, such as alkaline phosphatase or horseradish peroxidase. Such enzymatic markers are preferably heat stable, so as to survive the denaturation steps of the amplification

process. Primers may be indirectly labeled by incorporating a nucleotide covalently linked to a hapten or other molecule such as biotin to which a labeled avidin molecule may be bound, or digoxygenin, to which a labeled anti-digoxygenin antibody may be bound.

Primers may be labeled during chemical synthesis or the label may be attached after synthesis by methods known in the art. The method of labeling and the type of label is not critical to this invention.

It is contemplated that the probes or primers may be modified. For example the hydrolysis of a primer by 5' to 3' exonuclease associated with polymerase may be prevented by placing a phosphorothioate group between the last nucleotides of the 5' end of the primer. The extension of a primer by polymerase can be blocked by placing a dideoxynucleotide or a phosphate group at the 3' end. Alternatively, the extension of a primer may be blocked by placing an arabinosyl nucleotide at the 3' end of the primer which blocks extension by polymerase but allows ligation of the primer to another primer.

The term "the four different nucleotide bases" shall refer to deoxythymidine triphosphate (dTTP); deoxyadenosine triphosphate (dATP); deoxyguanosine triphosphate (dGTP); and deoxycytidine triphosphate (dCTP), when the context is DNA, but shall refer to uridine triphosphate (UTP); adenosine triphosphate (ATP); guanosine triphosphate (GTP); and cytidine triphosphate (CTP) when the context is RNA. Alternatively, dUTP, dITP, rITP or any other modified base may replace one of the four nucleotide bases or may be included along with the four nucleotide bases in the reaction mixture so as to be incorporated into the amplified strand. The amplification steps are conducted in the presence of at least the four deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP) or a modified nucleoside triphosphate to produce a DNA strand, or in the presence of the four ribonucleoside triphosphates (ATP,

CTP, GTP and UTP) or a modified nucleoside triphosphate to produce an RNA strand from extension of the oligonucleotide which acts as a primer.

Where the presence of a particular mutation or allele is to be detected by the methods of this invention, one of the oligonucleotide primers may comprise a set of oligonucleotide fragments, each differing in sequence and each labeled by a different method. That oligonucleotide fragment which is exactly complementary to the target DNA sequence will be detected by the presence of that label in the amplification products. In this case, each oligonucleotide fragment may be labeled as described above.

Utility

First Embodiment

In a first embodiment, the target nucleic acid is described as single stranded. However, this should be understood to include the case where the target is actually double stranded but is simply separated from its complementary strand prior to hybridization with probes/primers. Primers one and two, together, are substantially complementary to the target nucleic acid sequence and hybridize to adjacent regions of the target nucleic acid strand such that upon hybridization of the two primers to the target nucleic acid strand the 5' end of the first primer is adjacent to the 3' end of the second primer. The 3' end of the first primer is substantially complementary to the 5' end of the target nucleic acid sequence and the 5' end of the second primer is substantially complementary to the 3' end of the target nucleic acid sequence. The 5' end of the first primer is ligated to the 3' end of the second primer using ligase to create a fused amplification product in a double stranded complex. The fused primer is dissociated from the target nucleic acid.

The third primer is substantially complementary to all or at least a portion of the first primer and is similar to the 5' end of the target nucleic acid. The third primer may be smaller than the first primer or it may be larger than the first primer and also be substantially complementary to a portion of the second primer. The third primer is hybridized to the fused amplification product and extended by polymerase in the presence of at least four different nucleotide bases to form an extended amplification product which is substantially complementary to the fused amplification product. This comprises the first cycle.

Subsequently the double stranded complexes are dissociated. The oligonucleotide primers (1 and 2) are hybridized to the target nucleic acid sequence and the extended amplification product from the first cycle. Primer 3 is hybridized to the fused amplification product. Extension and ligation occur as before and the process can be repeated.

It is contemplated that the 3' end of the second primer may be modified to block the extension of the second primer by polymerase while still allowing ligation of the 3' end of the second primer to the 5' end of the first primer. Such modification may be, for example, the placement of an arabinosyl nucleotide at the 3' end of the second primer. Methods for the chemical synthesis of DNA oligomers containing cytosine arabinoside are known in the art (Beardsley, Nucl. Acid. Res. (1988) 16:9165-9176). Such a modification does not need to be removed prior to the ligation of the first and second primers.

Alternatively, it is also contemplated that the 5' end of the first primer can be modified to prevent the hydrolysis of the primer by a 5' to 3' exonuclease associated with a polymerase. Such a modification may be, for example, the placement of a phosphorothioate group between the last nucleotides of the 5' end of the first

primer. Methods for the chemical synthesis of phosphorothioate containing primers is known in the art (Ott and Eckstein, Biochemistry, (1987) 26:8237-8241). Such a modification does not need to be removed prior to ligation of the first and second primers.

It is further contemplated that extension of the first primer can be prevented without affecting the ligation of this primer by modifying the 3' end of the primer with a dideoxynucleotide or a phosphate group. The method for producing this modification is known in the art (Markiewicz and Wyrzykiewicz Nucl. Acid. Res. (1989) 17:7149-7158).

It has been found that the process can be conducted sequentially without isolation or purification of the products or removal of the excess reagents. Accordingly, this will allow the entire process to be conducted in a single reaction medium (e.g. a test tube).

It is understood that the single strand variation is a more specialized version of the double strand variation. If the target nucleic acid is double stranded some of the third primers will hybridize to the second complementary strand and the first and second primers will hybridize to the first strand. The extension and ligation from the first strand will proceed as described above. Some of the third primers will also be extended in a target specific manner complementary to the second strand. After dissociation of the extended third primer and the second strand, at least some of the first and second primers will hybridize to the extended third primer and at least some of the third primer will again hybridize to the second strand.

Where the target nucleic acid amplified by ligation of the first and second primers and extension of the third primer is to be detected, one or all of these primers may be labeled using a marker as described above to render the amplified target nucleic acid detectable or by conducting the extension of the third primer in the presence of a labeled base, or a base which is activated for labeling.

Alternatively, where the target nucleic acid is double stranded, both amplified strands may be labeled with different detectable markers: the first strand may be labeled by labeling the third primer with a particular marker; and the second strand may be labeled by labeling the first and/or second primer.

In the case where the presence of a particular point mutation or allele is to be detected, one primer comprising a mixture of oligonucleotides is added to the nucleic acid sample. Each oligonucleotide may be labeled with different, separately detectable markers, so that information regarding the presence of a particular mutation or allele may be obtained in a single step. The oligonucleotide which is exactly complementary to the target sequence will be included in the amplification product whereas the other oligonucleotides will not and its presence detected by determining which label is included in the product.

The amplification reaction is optimally conducted with an excess of primers at a ratio of oligonucleotide primers:target of approximately 10^7 to 10^3:1, more preferably approximately 10^4:1. It is contemplated that adjusting the molarity of the primers will maximize the efficiency of the process.

The buffer used for amplification is preferably in a pH range of about 7.5-8.5, more preferably about 8-8.5, and most preferably about 8.0.

Second Embodiment

In a second embodiment, the target nucleic acid is described to be single stranded. However, this should be understood to include the case where the target is actually double stranded, but is simply separated from its complementary strand prior to hybridization with the probes/primers.

The target nucleic acid is hybridized to two primers.

The first primer is substantially complementary to the 5' end of the target nucleic acid sequence and the second primer is substantially complementary to the 3' end of the target nucleic acid sequence. The primers (primers one and two) hybridize to regions of the target nucleic acid strand such that upon hybridization of the two primers to the target nucleic acid strand the 5' end of the first primer is spaced from the 3' end of the second primer. The size of the space or gap between the primers is determined by the ability of a polymerase or transcriptase to extend the second primer such that the newly added 3' end of the second primer is directly adjacent to the 5' end of the first primer. Preferably, but not necessarily, the size of the gap or space is sufficiently long such that at least four different nucleotides would be required by the polymerase or transcriptase in order to extend the second primer to "fill in" the gap.

The 3' end of the second primer is extended by polymerase or transcriptase in the presence of the four nucleotide bases. The 5' end of the first primer is then ligated to the new 3' end of the second extended primer to form a double-stranded complex comprising the target nucleic acid and an extended fused primer.

The double stranded complex is dissociated and a third primer is hybridized to the extended fused primer. The third primer is substantially complementary to all or a portion of the first primer and is similar to the 5' end of the target nucleic acid sequence. The 3' end of the third primer is extended by polymerase or transcriptase to form a double-stranded complex. The double-stranded complex is dissociated and the cycle repeated until the target nucleic acid is amplified.

It is contemplated that the process can be conducted sequentially without isolation or purification of the products or removal of the excess reagents. Accordingly, this will allow the entire process to be conducted in a

single reaction medium (e.g. a test tube). Further, because the gap between the primers can be any size, the method is not limited to a particular DNA sequence and extension of the third primer can proceed in the presence of four nucleotides.

It is understood that the single strand variation is a more specialized case of the double strand variation wherein there are four primers and the first and second primers are substantially complementary to the first strand of the target nucleic acid and the third and fourth primers are substantially complementary to the second strand of the target nucleic acid. The third primer being substantially complementary to at least a portion of the first primer and the fourth primer being substantially complementary to at least a portion of the second primer. The extension and ligation of the third and fourth primers occurs as described above for the first and second primers.

It is contemplated that the 5' end of the first primer (and the 5' end of the fourth primer, where the nucleic acid is double stranded) can be modified to prevent the hydrolysis of the primer by a 5' to 3' exonuclease associated with the polymerase. Such a modification may be, for example, the placement of a phosphorothioate group between the last nucleotides of the 5' end of the first or fourth primers. Methods for the chemical synthesis of phosphorothioate containing primers is known in the art (Ott and Eckstein, Biochemistry, (1987) 26:8237-8241). Such a modification does not need to be removed prior to ligation of the first and second primers.

It is further contemplated that extension of the first and fourth primers can be prevented without affecting the ligation of these primers by modifying the 3' end of the primers with a dideoxynucleotide or a phosphate group. This method of producing this modification is known in the art (Markiewicz and Wyrzykiewicz Nucl. Acid. Res. (1989) 17:7149-7158).

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Where the target nucleic acid amplified is to be detected, one or all of these primers may be labeled as described above to render the amplified strand detectable. Alternatively the strand may be labeled by conducting the extension of the second or third primer in the presence of a labeled base, or a base which is activated for labeling.

In the case where one primer comprises a mixture of oligonucleotides to detect the presence of a particular sequence, each of the oligonucleotides may be labeled with different, separately detectable markers, so that information regarding each mutation may be obtained in a single step.

The amplification reaction is optimally conducted with an excess of primers at a ratio of oligonucleotide primers:target of approximately 10^7 to 10^3:1, more preferably approximately 10^4:1. It is contemplated that adjustment of the molarity of the primers will maximize the efficiency of the process.

The buffer used for amplification is preferably in a pH range of about 7.5-8.5, more preferably about 8-8.5, and most preferably about 8.0.

If the target nucleic acid is double stranded, the strands should be separated so that they can be used individually. This separation can be accomplished by any suitable denaturation method including physical, chemical or enzymatic means, each of which are well known in the art.

In either of the above embodiments, the amplification reaction will involve a series of steps. The reaction may be either a two step process [i.e. 1) hybridization/extension/ligation followed by 2) denaturation] or a three step process [1) hybridization; 2) extension/ligation and 3) denaturation). These steps may be carried out manually, but they are preferably conducted in an automated thermal cycler.

Hybridization is generally conducted at a temperature of

approximately 50-75°C for a period of 0.5-2 minutes, more preferably at 60-70°C for a period of 1-1.5 minutes, and most preferably at about 63-68°C for about 1 minute. The extension/ligation or the hybridization/extension/ligation steps are generally conducted at a temperature of approximately 60-80°C for a period of 0.5-5 minutes, more preferably at 68-78°C for a period of 2-4 minutes.

The conditions and reagents which make possible the preferred enzymatic ligation step are generally known to those of ordinary skill in the art and depend directly on the type of ligase used. The "ligating enzyme" may be any enzyme known in the art to ligate nucleic acid sequences, including T4 ligase, but it is preferably a ligase stable at temperatures of approximately 0-95°C, such as AMPLIGASE (Epicentre Technologies, Madison Wisconsin) and Taq ligase (New England Biolabs, Beverly, Massachusetts). Absent a thermally stable ligase, the ligase must be added again each time the cycle is repeated. Approximately at least 1 units of ligating enzyme/picomole of oligonucleotide is used. One unit is defined as the amount required to catalyze the ligation of 50% of the cos sites in one microgram of BstE II digested bacteriophage λ DNA in fifteen minutes at 45°C.

The "polymerase" may be any enzyme capable of polymerizing an RNA or DNA strand, including E. coli DNA polymerase I, the Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, RNA polymerase or reverse transcriptase. In general, the primer is extended by the polymerase in a target dependent manner, for example, under conditions such that a nucleic acid strand is formed complementary to the nucleic acid sequence to which the primer is hybridized. Preferably, the polymerizing enzyme is stable at temperatures of approximately 0-95°C, such as Taq DNA polymerase (Perkin-Elmer Corporation, Norwalk, Connecticut). Absent a thermally stable polymerase, the

polymerase must be added again each time the cycle is repeated. At least approximately 0.05 units of polymerizing enzyme (as defined by the manufacturer)/picomole of oligonucleotide is used.

Extension of a primer by polymerase or transcriptase proceeds in a 5' to 3' direction and requires the addition in adequate amounts of at least the four nucleotide bases in the reaction mixture.

After extension of the primers, it is necessary to separate the nucleic acid strands. The strand separation can be accomplished by any suitable denaturing method including well-known physical, chemical or enzymatic means. For example, one physical method of separating the strands of the nucleic acid involves heating the nucleic acid until it is completely denatured. Typical heat denaturation is generally conducted at a temperature of approximately 85-110°C, more preferably at 90-100°C, and most preferably at about 92-96°C for a period of at least about 0.5 minutes. One skilled in the art would understand how to modify the temperatures and times so as to optimize the results obtained with different oligonucleotide primers. Alternatively, denaturation can be achieved by other methods known in the art. One such method is by the introduction of a nucleic acid-unwinding enzyme such as helicase.

The reaction is stopped by any method known in the art, such as with a buffer containing a high percentage of denaturant such as formamide, EDTA or by freezing. The products can then be analyzed by any method, but electrophoresis on a polyacrylamide gel is preferable. Preferably, the samples are boiled before loading on the gel to eliminate any secondary structures. The gel may then be dried and placed against autoradiographic film or phosphor screen when the oligonucleotides or amplified strands contain radioactive nuclides. The gel may also be blotted and probed with a probe specific to the region

amplified.

The primer may be labeled with a detectable marker by any method known in the art. A preferred method for labeling primers is by end labeling. Primers may be labeled during chemical synthesis by substitution of the ^{31}P atoms in the phosphate groups with ^{32}P. The substituted nucleotide may be directly labeled or contain a linker arm for attaching a label, or may be attached to a hapten or other molecule to which a labeled binding molecule may bind (Boehringer Mannheim, Indianapolis, Indiana). Suitable direct labels include radioactive labels such as ^{32}P, ^{3}H, and ^{35}S and non-radioactive labels such as fluorescent markers, such as fluorescein, Texas Red, AMCA blue, lucifer yellow, rhodamine, and the like; cyanin dyes which are detectable with visible light; enzymes and the like.

Fluorescent markers may alternatively be attached to nucleotides with activated linker arms. Primers may be indirectly labeled by the methods disclosed above, by incorporating a nucleotide covalently linked to a hapten or other molecule such as biotin or digoxygenin, and performing a sandwich hybridization with a labeled antibody directed to that hapten or other molecule, or in the case of biotin, with avidin conjugated to a detectable label. Antibodies and avidin may be conjugated with a fluorescent marker, or with an enzymatic marker such as alkaline phosphatase or horseradish peroxidase to render them detectable. Conjugated avidin and antibodies are commercially available from companies such as Vector Laboratories (Burlingame, California) and Boehringer Mannheim (Indianapolis, Indiana).

The enzyme can be detected through a colorimetric reaction by providing a substrate and/or a catalyst for the enzyme. In the presence of various catalysts, different colors are produced by the reaction, and these colors can be visualized to separately detect multiple probes. Any substrate and catalyst known in the art may be used.

Preferred catalysts for alkaline phosphatase include 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT). The preferred substrate for horseradish peroxidase is diaminobenzoate (DAB).

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

Abbreviations:

ATP	-	adenosine triphosphate
dATP	-	deoxyadenosine triphosphate
CTP	-	cytidine triphosphate
dCTP	-	deoxycytidine triphosphate
GTP	-	guanosine triphosphate
dGTP	-	deoxyguanosine triphosphate
dTTP	-	thymidine triphosphate
UTP	-	uridine triphosphate
NTP	-	nucleoside triphosphate
nmole (nM)	-	nanomole
pmole (pM)	-	picomole
mmole (mM)	-	millimole
(µM)	-	micromole
ng	-	nanogram
µg	-	microgram
bis	-	bisacrylamide (N, N'-methylenebis-acrylamide)
5'	-	the 5' position in the pentose
3'	-	the 3' position in the pentose

EXAMPLE 1

Preparation of Target DNA

A 889 basepair region of the multidrug resistance gene (MDR-1) (Figure 4, SEQ ID NO:1) was selected as a target DNA for the system. The MDR-1 gene is available from the American Type Culture Collection, ATCC No. 65704. The target DNA was prepared by the standard polymerase chain

reaction with
Primer A (SEQ ID. NO:2) 5'-AGGTTAGTACCAAAGAGGCTCTGG-3' and
Primer B (SEQ ID NO:3) 5'-ACTAACAGAACATCCTCAAAGCTC-3'
based on the known sequence of the gene. The PCR reaction mixture comprised 1mM Tris HCl (pH 8.4), 5 mM KCl, 1.2 mM $MgCl_2$, 0.8 mM of each dNTP, 1 µM of Primer A, 1 µM of Primer B, 1 ng of template DNA, 2.5 units of Amplitaq™ DNA polymerase (Perkin Elmer Cetus Corporation, Norwalk, Connecticut). The reaction mixture was heated at 94°C for 6 min., and then put through the following cycle 30 times: 94°C for 1 min, 65°C for 45 sec., and 72°C for 3 min. The final polymerization was done at 72°C for 10 min.

20 µg of DNA was digested with 40 units of *RsaI* restriction endonuclease at 37°C for 2 hours under the conditions recommended for the enzyme. An aliquot was run on an agarose gel to confirm that the DNA was completely digested. DNA was then extracted sequentially with equal volumes of phenol, phenol-chloroform (1:1) and chloroform, and then precipitated with two volumes of ethanol. The DNA pellet was suspended in deionized water and the concentration determined by measuring the optical density at 260 nm.

EXAMPLE 2

Preparation of Oligonucleotides

Deoxynucleotide oligomers were synthesized on Milligen/Biosearch Cyclone Plus DNA Synthesizers [Millipore Corporation, Bedford, Massachusetts] using beta-cyanoethyl phosphoramidite chemistry. All reagents for oligonucleotide synthesis were purchased from Millipore Corporation [Bedford, Massachusetts].

Oligonucleotides having the following sequences were synthesized:
Oligo 1 (SEQ ID NO. 4):
5' CAACATTTTC ATTTCAACAA CTCC 3'
Oligo 2 (SEQ ID NO. 5):

5' TTCTTTCTTA TCTTTCAGTG CTTGTCCAGA 3'

Oligo 3 (SEQ ID NO. 6):

5' GGAGTTGTTG AAATGAAAAT GTTGTC 3'

After the specified sequence had been assembled, a 60 minute room temperature treatment with ammonium hydroxide was used to cleave the oligonucleotide from the support. The oligonucleotide was incubated with ammonium hydroxide at 55°C overnight treatment to remove the protecting groups. Ammonium hydroxide was evaporated to dryness in a Speedvac Concentrator [Savant Instruments, Inc., Farmingdale, New York]. The oligonucleotide was suspended in deionized water and extracted three times with an equal volume of water-saturated N-butanol. Any traces of N-butanol left were removed by evaporation in a Speedvac Concentrator. The concentration of oligonucleotide was determined by measuring optical density at 260 nm in a spectrophotometer.

EXAMPLE 3

Phosphorylation of oligonucleotides

Each oligonucleotide was phosphorylated at the 5' end with ATP and T4 polynucleotide kinase. The reaction mixture (100 µl) contained 2 nmoles of each oligonucleotide 50 mM Tris HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine hydrochloride, 0.1 mM EDTA, 1 mM ATP and 50 units of T4 polynucleotide kinase (GIBCO BRL, Gaithersburg, Maryland). After 1 hour at 37°C, the enzyme was inactivated by heating at 65°C for 10 minutes.

EXAMPLE 4

^{32}P Labeling of oligonucleotides

Oligonucleotides (20 pmoles) were labeled with ^{32}P at their 5' end in 60 µl of 50 mM Tris HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine hydrochloride, 0.1 mM EDTA and 200 µCi of [γ-^{32}P] ATP (3000 Ci/mmole = 67 pmoles

of ATP; NEN Research Products Div. of Dupont, Boston, Massachusetts). The reaction was started by adding 20 units of T4 polynucleotide kinase (GIBCO BRL, Gaithersburg, Maryland) and incubated at 37°C for 1 hour. T4 polynucleotide kinase was heat inactivated at 65°C for 10 minutes.

EXAMPLE 5

Amplification of DNA

Oligonucleotides 1, 2 and 3 at a final concentration of 0.2 µM were incubated in the presence or absence of target DNA (0.5 fmole = 3 x 10^8 molecules) in 20 µl of 25 mM Tris HCl pH 8.0, 10 mM KCl, 2 mM $MgCl_2$, 10 mM DTT, 2 mM NAD^+ and 50 µM of dATP, dGTP, dCTP and dTTP. The stock solution of dNTP's was maintained at -20°C.

Three different experiments were performed. In each case, only one oligonucleotide was labeled. 15 units of Taq ligase (New England Biolabs, Beverly, Massachusetts) and 1 unit of Amplitaq™ DNA Polymerase (Perkin-Elmer Corporation, Norwalk, Connecticut) were added and the mixture was overlaid with a drop of mineral oil. Reactions were incubated in a single reaction medium in an Ericomp™ Thermal Cycler (Ericomp Incorporation, San Diego, California) at 94°C for 6 minutes. The reaction mixture was incubated for 1 minute at 94°C, and 4 minutes at 65°C, with this cycle being repeated 30 times.

The product formation was followed independently using each ^{32}P-labeled oligonucleotide. The reaction was stopped by adding 13 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were stored at -20°C until analyzed by electrophoresis.

EXAMPLE 6

Separation of the amplification products

The products of the amplification reaction were separated on an 8% polyacrylamide gel (acrylamide:bis; 19:1) containing 8M urea in 100 mM Tris Borate pH 8.3, 2 mM EDTA. A BRL sequencing gel apparatus model S2 (BRL, Gaithersburg, Maryland) was used to run the gel.

Samples (4 µl) were denatured by boiling before loading on the gel. Electrophoresis was performed at a constant 60 watts for 2 hours. The gel was dried and exposed to a Phospho Screen™ (Molecular Dynamics, Sunnyvale, California) and analyzed by a Phosphor Imager™ (Molecular Dynamics, Sunnyvale , California).

Figure 2 is a printout from a Phosphor Imager scan of the samples amplified by the method described in Example 5. In Lane 1 the reaction mixture contained labeled Oligo 1 and unlabeled Oligo 2 and 3. In Lane 2 the reaction mixture was the same as in Lane 1 with the addition of target DNA. The amplified DNA band is indicated with an arrow. In Lane 3 the reaction contained labeled Oligo 2 and unlabeled Oligo 1 and 3. In Lane 4 the reaction mixture was the same as for Lane 3 with the addition of target DNA. In Lane 5 the reaction mixture contained unlabeled Oligo 1 and 2 and labeled Oligo 3. In Lane 6, the reaction mixture was the same as in Lane 5 with the addition of target DNA. It can be seen that amplification does not occur in the absence of the target DNA and that amplification can be detected by labeling any of the oligonucleotides.

EXAMPLE 7

Embodiment 2

Deoxynucleotides are synthesized on Milligen/Biosearch Cyclone Plus™ DNA synthesizers (Millipore Corporation, Bedford Massachusetts) using beta-cyanoethyl phosphoramidite chemistry as described in Example 2. The synthesis of oligonucleotides 1 and 3 was previously described in Example 2.

Oligonucleotides having the following sequences are synthesized:

Oligo 4 (SEQ ID NO:7)

5' GTTCGGAAGT TTTCTATTGC TTCAGTAGCG 3'

Oligo 5 (SEQ ID NO:8)

5' CTACTGAAGC AATAGAAAAC TTCCGAAC 3'

The oligonucleotides are either phosphorylated at the 5' end with ATP and T4 polynucleotide kinase as described in Example 3 or labeled with ^{32}P at their 5' end as described in Example 4.

The target DNA is prepared as described in Example 1.

Phosphorylated oligonucleotides at a final concentration of 0.2 µM are incubated in the presence of target DNA (0.5 fmole = 3 x 10^8 molecules) in 20 µl of 25 mM Tris HCl pH 8.0, 10 mM KCl, 2 mM $MgCl_2$, 10 mM DTT, 2 mM NAD^+ and 50 µM of dATP, dCTP, dGTP and dTTP. The stock solution of dNTP's are maintained at -20°C.

15 units of Taq ligase (New England Biolabs, Beverly, Massachusetts) and 1 unit of Amplitaq™ DNA polymerase (Perkin-Elmer Corporation, Norwalk, Connecticut) are added and the mixture is overlaid with a drop of mineral oil. Reactions are incubated in a single reaction medium in an Ericomp Thermal Cycler™ (Ericomp Incorporation, San Diego, California) at 94°C for 6 minutes. Then the reaction mixture is incubated for 1 minute at 94°C, and 4 minutes at 65°C, with this cycle being repeated 30 times.

The reaction is stopped by adding 13 µl of stop solution (95% v/v formamide, 20 mM EDTA, 0.05% w/v bromophenol blue, 0.05% w/v xylene cyanol FF). Samples are stored at -20°C until analyzed by electrophoresis.

The products of the amplification reaction are separated as described in Example 6.

Although only preferred embodiments of the invention are specifically illustrated and described above, it will be appreciated that many modifications and variations of the present invention are possible in light of the above

teachings and within the purview of the appended claims without departing from the spirit and intended scope of the invention.

IC ACID

OX 1404

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BHATNAGAR, SATISH K.
GEORGE JR., ALBERT L.

(ii) TITLE OF INVENTION: METHODS FOR AMPLIFYING NUCLEIC ACID SEQUENCES

(iii) NUMBER OF SEQUENCES: 8

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/010,433
(B) FILING DATE: 27-JAN-1993
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: SWISS, GERALD F.
(B) REGISTRATION NUMBER: 30,113
(C) REFERENCE/DOCKET NUMBER: 020160-103

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: 415-854-8275

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2726 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

1560	GATTGAGAAA GCTGTCAAGG AAGCCAATGC CTATGACTTT ATCATGAAAC TGCCTCATAA	60
1620	ATTTGACACC CTGGTTGGAG AGAGAGGGGC CCAGTTGAGT GGTGGGCAGA AGCAGAGGAT	120
1680	CGCCATTGCA CGTGCCCTGG TTCGCAACCC CAAGATCCTC CTGCTGGATG AGGCCACGTC	180
1740	AGCCTTGGAC ACAGAAAGCG AAGCAGTGGT TCAGGTGGCT CTGGATAAGG CCAGAAAAGG	240
1800	TCGGACCACC ATTGTGATAG CTCATCGTTT GTCTACAGTT CGTAATGCTG ACGTCATCGC	300
1860	TGGTTTCGAT GATGGAGTCA TTGTGGAGAA AGGAAATCAT GATGAACTCA TGAAAGAGAA	360
1920	AGGCATTTAC TTCAAACTTG TCACAATGCA GACAGCAGGA AATGAAGTTG AATTAGAAAA	420
1980	TGCAGCTGAT GAATCCAAAA GTGAAATTGA TGCCTTGGAA ATGTCTTCAA ATGATTCAAG	480
2040	ATCCAGTCTA ATAAGAAAAA GATCAACTCG TAGGAGTGTC CGTGGATCAC AAGCCCAAGA	540
2100	CAGAAAGCTT AGTACCAAAG AGGCTCTGGA TGAAAGTATA CCTCCAGTTT CCTTTTGGAG	600
2160	GATTATGAAG CTAAATTTAA CTGAATGGCC TTATTTTGTT GTTGGTGTAT TTTGTGCCAT	660
2220	TATAAATGGA GGCCTGCAAC CAGCATTTGC AATAATATTT TCAAAGATTA TAGGGGTTTT	720
2280	TACAAGAATT GATGATCCTG AAACAAAACG ACAGAATAGT AACTTGTTTT CACTATTGTT	780
2340	TCTAGCCCTT GGAATTATTT CTTTTATTAC ATTTTTCCTT CAGGGTTTCA CATTTGGCAA	840
2400	AGCTGGAGAG ATCCTCACCA AGCGGCTCCG ATACATGGTT TTCCGATCCA TGCTCAGACA	900
2460	GGATGTGAGT TGGTTTGATG ACCCTAAAAA CACCACTGGA GCATTGACTA CCAGGCTCGC	960
2520	CAATGATGCT GCTCAAGTTA AAGGGGCTAT AGGTTCCAGG CTTGCTGTAA TTACCCAGAA	1020
2580	TATAGCAAAT CTTGGGACAG GAATAATTAT ATCCTTCATC TATGGTTGGC AACTAACACT	1080
2640	GTTACTCTTA GCAATTGTAC CCATCATTGC AATAGCAGGA GTTGTTGAAA TGAAAATGTT	1140
2700	GTCTGGACAA GCACTGAAAG ATAAGAAAGA ACTAGAAGGT GCTGGGAAGA TCGCTACTGA	1200
2726	AGCAATAGAA AACTTCCGAA CCGTTGTTTC TTTGACTCAG GAGCAGAAGT TTGAACATAT	1260
	GTATGCTCAG AGTTTGCAGG TACCATACAG AAACTCTTTG AGGAAAGCAC ACATCTTTGG	1320
	AATTACATTT TCCTTCACCC AGGCAATGAT GTATTTTTCC TATGCTGGAT GTTTCCGGTT	1380
	TGGAGCCTAC TTGGTGGCAC ATAAACTCAT GAGCTTTGAG GATGTTCTGT TAGTATTTTC	1440
	AGCTGTTGTC TTTGGTGCCA TGGCCGTGGG GCAAGTCAGT TCATTTGCTC CTGACTATGC	1500

CAAAGCCAAA ATATCAGCAG CCCACATCAT CATGATCATT GAAAAAACCC CTTTGATTGA 1560

CAGCTACAGC ACGGAAGGCC TAATGCCGAA CACATTGGAA GGAAATGTCA CATTTGGTGA 1620

AGTTGTATTC AACTATCCCA CCCGACCGGA CATCCCAGTG CTTCAGGGAC TGAGCCTGGA 1680

GGTGAAGAAG GGCCAGACGC TGGCTCTGGT GGGCAGCAGT GGCTGTGGGA AGAGCACAGT 1740

GGTCCAGCTC CTGGAGCGGT TCTACGACCC CTTGGCAGGG AAAGTGCTGC TTGATGGCAA 1800

AGAAATAAAG CGACTGAATG TTCAGTGGCT CCGAGCACAC CTGGGCATCG TGTCCCAGGA 1860

GCCCATCCTG TTTGACTGCA GCATTGCTGA GAACATTGCC TATGGAGACA ACAGCCGGGT 1920

GGTGTCACAG GAAGAGATCG TGAGGGCAGC AAAGGAGGCC AACATACATG CCTTCATCGA 1980

GTCACTGCCT AATAAATATA GCACTAAAGT AGGAGACAAA GGAACTCAGC TCTCTGGTGG 2040

CCAGAAACAA CGCATTGCCA TAGCTCGTGC CCTTGTTAGA CAGCCTCATA TTTTGCTTTT 2100

GGATGAAGCC ACGTCAGCTC TGGATACAGA AAGTGAAAAG GTTGTCCAAG AAGCCCTGGA 2160

CAAAGCCAGA GAAGGCCGCA CCTGCATTGT GATTGCTCAC CGCCTGTCCA CCATCCAGAA 2220

TGCAGACTTA ATAGTGGTGT TTCAGAATGG CAGAGTCAAG GAGCATGGCA CGCATCAGCA 2280

GCTGCTGGCA CAGAAAGGCA TCTATTTTTC AATGGTCAGT GTCCAGGCTG GAACAAAGCG 2340

CCAGTGAACT CTGACTGTAT GAGATGTTAA ATACTTTTTA ATATTTGTTT AGATATGACA 2400

TTTATTCAAA GTTAAAAGCA AACACTTACA GAATTATGAA GAGGTATCTG TTTAACATTT 2460

CCTCAGTCAA GTTCAGAGTC TTCAGAGACT TCGTAATTAA AGGAACAGAG TGAGAGACAT 2520

CATCAAGTGG AGAGAAATCA TAGTTTAAAC TGCATTATAA ATTTTATAAC AGAATTAAAG 2580

TAGATTTTAA AAGATAAAAT GTGTAATTTT GTTTATATTT TCCCATTTGG ACTGTAACTG 2640

ACTGCCTTGC TAAAAGATTA TAGAAGTAGC AAAAAGTATT GAAATGTTTG CATAAAGTGT 2700

CTATAATAAA ACTAAACTTT CATGTG 2726

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGGTTAGTAC CAAAGAGGCT CTGG 24

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACTAACAGAA CATCCTCAAA GCTC 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAACATTTTC ATTTCAACAA CTCC 24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTCTTTCTTA TCTTTCAGTG CTTGTCCAGA 30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAGTTGTTG AAATGAAAAT GTTGTC 26

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTTCGGAAGT TTTCTATTGC TTCAGTAGCG 30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTACTGAAGC AATAGAAAAC TTCCGAAC 28

WHAT IS CLAIMED IS:

1. A process for amplifying enzymatically a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, comprising the steps of:

a) selecting the target nucleic acid sequence;

b) providing primers, said primers comprising a first primer which is substantially complementary to a first segment at a first end of the target nucleic acid sequence and a second primer which is substantially complementary to a second segment at a second end of the target nucleic acid sequence and whose 3' end is adjacent to the 5' end of the first primer and a third primer which is similar to the first end of the target nucleic acid sequence and which is substantially complementary to at least a portion of said first primer;

c) providing at least four different nucleotide bases;

d) hybridizing said first and second primers to the target nucleic acid sequence in a target dependent manner to form a primer-target complex;

e) ligating under conditions such that the adjacent 5' end of the first primer and the 3' end of the second primer will ligate to form a fused amplification product substantially complementary to said target nucleic acid sequence;

f) dissociating said fused amplification product from said target nucleic acid sequence;

g) hybridizing said third primer to said fused amplification product;

h) extending said third primer in the presence of the nucleotide bases under conditions such that an extended amplification product is formed substantially complementary to said fused amplification product; and

i) dissociating the extended amplification product from the fused amplification product.

2. The process of Claim 1, wherein the target nucleic acid is single stranded.

3. The process of Claim 1, wherein steps (d) through (i) are repeated at least once.

4. The process of Claim 1, wherein the target nucleic acid is DNA.

5. The process of Claim 1, wherein the target nucleic acid is RNA.

6. The process of Claim 1, wherein step (e) is conducted in the presence of a ligating enzyme.

7. The process of Claim 6, wherein the ligating enzyme is T4 DNA ligase.

8. The process of Claim 6, wherein the ligating enzyme is stable at 0-95°C.

9. The process of Claim 8, wherein the ligating enzyme is Taq ligase.

10. The process of Claim 1, wherein step (h) is conducted in the presence of a polymerase.

11. The process of Claim 10, wherein step (h) is conducted in the presence of a polymerase selected from the group consisting of E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, and T4 DNA polymerase.

12. The process of Claim 10, wherein step (h) is conducted in the presence of a polymerase which is stable

at temperatures of 0-95°C.

13. The process of Claim 12, wherein the agent for polymerization is Taq DNA polymerase.

14. The process of Claim 1, wherein the target nucleic acid sequence contains at least one deletion or mutation that causes a genetic disease.

15. The process of Claim 1, wherein the target nucleic acid sequence is contained in a pathogenic organism, virus or oncogene.

16. The process of Claim 1, wherein one of said primers comprises two or more different oligonucleotides, one of said oligonucleotides having a sequence exactly complementary to said target nucleic acid sequence.

17. The process of Claim 1, wherein the target nucleic acid is double stranded nucleic acid comprising a first and second strand wherein said first and second primers are substantially complementary to said first strand and said third primer is substantially complementary to said second strand and said first and second strands are dissociated prior to step (d).

18. The process of Claim 1, wherein each of the steps is conducted sequentially without isolation or purification of the products.

19. The process of Claim 18 wherein each of the steps is conducted in a single reaction medium.

20. The process of Claim 1, wherein the 5' end of the first primer comprises a phosphorothioate group.

21. The process of Claim 1, wherein the 3' end of the second primer comprises an arabinosyl nucleotide.

22. A process for detecting enzymatically a mutation or an allele in a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, comprising the steps of:

a) selecting the target nucleic acid sequence;

b) providing primers, said primers comprising a first primer which is substantially complementary to a first segment at a first end of the target nucleic acid sequence and a second primer which is substantially complementary to a second segment at a second end of the target nucleic acid sequence and whose 3' end is adjacent to the 5' end of the first primer and a third primer which is similar to the first end of the target nucleic acid sequence and which is substantially complementary to at least a portion of said first primer, wherein one of said primers comprises two or more different oligonucleotides, one of said oligonucleotides having a sequence exactly complementary to said target nucleic acid sequence wherein each oligonucleotide is labeled with a different label;

c) providing at least four different nucleotide bases;

d) hybridizing said first and second primers to the target nucleic acid sequence in a target dependent manner to form a primer-target complex;

e) ligating under conditions such that the adjacent 5' end of the first primer and the 3' end of the second primer will ligate to form a fused amplification product substantially complementary to said target nucleic acid sequence;

f) dissociating said fused amplification product from said target nucleic acid sequence;

g) hybridizing said third primer to said fused amplification product;

h) extending said third primer in the presence of the nucleotide bases under conditions such that an extended amplification product is formed substantially complementary to said fused amplification product;

i) dissociating the extended amplification product from the fused amplification product; and

j) determining which labeled primer is contained within the fused amplification product or the extended amplification product.

23. A process for amplifying enzymatically a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids comprising the steps of:

a) selecting the target nucleic acid sequence;

b) providing primers, said primers comprising a first primer which is substantially complementary to a first segment at a first end of the target nucleic acid sequence and a second primer which is substantially complementary to a second segment at a second end of the target nucleic acid sequence said second segment being spaced a number of nucleotides from said first segment and a third primer which is similar to the first end of the target nucleic acid sequence and which is substantially complementary to at least a portion of said first primer;

c) providing at least four nucleotide bases;

d) hybridizing said first and second primers to the target nucleic acid sequence in a target dependent manner to form a primer-target complex;

e) extending a 3' end of the second primer in the presence of the nucleotide bases under conditions such that an extended second primer is formed wherein the 3' end of the extended second primer terminates at a base adjacent to a 5' end of the first primer;

f) ligating the ends of the first and second extended primers under conditions such that said first and said extended second primers will form a fused

amplification product substantially complementary to said target nucleic acid sequence;

g) dissociating said fused amplification product from said target nucleic acid sequence;

h) hybridizing said third primer to said fused amplification product;

i) extending said third primer in the presence of the nucleotide bases under conditions such that an extended amplification product is formed substantially complementary to said fused amplification product; and

j) dissociating the extended amplification product from the fused amplification product.

24. The process of Claim 23, wherein steps (d) and (j) are repeated at least once.

25. The process of Claim 23, wherein the target nucleic acid is double stranded nucleic acid comprising a first and second strand wherein said first and second primers are substantially complementary to said first strand and said third primer is substantially complementary to said second strand and said first and second strands are dissociated prior to step (d).

26. The process of Claim 25, further comprising a fourth primer wherein the fourth primer is substantially complementary to said second target nucleic acid strand and said fourth primer is substantially complementary to said second primer.

27. The process of Claim 26, wherein the nucleic acid is denatured by heating.

28. The process of Claim 23, wherein the nucleic acid is DNA.

29. The process of Claim 23, wherein the nucleic acid is RNA.

30. The process of Claim 23, wherein step (f) is conducted in the presence of a ligating enzyme.

31. The process of Claim 30, wherein the ligating enzyme is T4 DNA ligase.

32. The process of Claim 30, wherein the ligating enzyme is stable at 0-95°C.

33. The process of Claim 32, wherein the ligating enzyme is Ampligase.

34. The process of Claim 23 wherein steps (e) and (i) are conducted in the presence of polymerase.

35. The process of Claim 34, wherein steps (e) and (i) are conducted in the presence of a polymerase selected from the group consisting of E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I and T4 DNA polymerase.

36. The process of Claim 34, wherein steps (e) and (i) are conducted in the presence of a polymerase which is stable at 0-95°C.

37. The process of Claim 36, wherein the polymerase is Taq DNA polymerase.

38. The process of Claim 23, wherein the target nucleic acid sequence contains at least one deletion or mutation that causes a genetic disease.

39. The process of Claim 23, wherein the target nucleic acid sequence is contained in a pathogenic organism, virus

or oncogene.

40. The process of Claim 23, wherein one of said primers comprises two or more oligonucleotides, one of said oligonucleotides having a sequence exactly complementary to said target nucleic acid.

41. The process of Claim 23, wherein each of the steps is conducted sequentially without isolation or purification of the products.

42. The process of Claim 41, wherein each of the steps is conducted in a single reaction medium.

43. The process of Claim 23, wherein the 5' end of the first primer comprises an phosphorothioate group.

44. The process of Claim 26, wherein the 5' end of the fourth primer comprises an phosphorothioate group.

45. A process for detecting enzymatically a mutation or an allele in a target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids comprising the steps of

a. selecting the target nucleic acid sequence;

b. providing primers, said primers comprising a first primer which is substantially complementary to a first segment at a first end of the target nucleic acid sequence and a second primer which is substantially complementary to a second segment at a second end of the target nucleic acid sequence said second segment being spaced from said first segment and a third primer which is similar to the first end of the target nucleic acid sequence and which is substantially complementary to at least a portion of said first primer wherein one of said primers comprises two or more different oligonucleotides,

one of said oligonucleotides having a sequence exactly complementary to said target nucleic acid sequence wherein each oligonucleotide is labeled with a different label;

c. providing at least four nucleotide bases;

d. hybridizing said first and second primers to the target nucleic acid sequence in a target dependent manner to form a primer-target complex;

e. extending a 3' end of the second primer in the presence of the nucleotide bases under conditions such that an extended second primer is formed wherein the 3' end of the extended second primer is adjacent to a 5' end of the first primer;

f. ligating the ends of the first and second primers under conditions such that said first and said extended second primers will form a fused amplification product complementary to said target nucleic acid sequence;

g. dissociating said fused amplification product from said target nucleic acid sequence;

h. hybridizing said third primer to said fused amplification product;

i. extending said third primer in the presence of the nucleotide bases under conditions such that an extended amplification product is formed complementary to said fused amplification product; and

j. dissociating the extended amplification product from the fused amplification product; and

k. determining which labeled primer is contained within the fused amplification product or the extended amplification product.

46. A kit for amplifying at least one target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, in accordance with Claim 1, comprising:

a. first, second and third primers;

b. a ligating enzyme;

c. a polymerizing enzyme; and

d. at least four nucleotides.

47. The kit of Claim 46 further comprising:

e. a detectable marker attached to one of said primers.

48. The kit of Claim 46, further comprising:

e. a buffer suitable for the ligation and polymerization reactions.

49. A kit for amplifying at least one target nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, in accordance with Claim 23, comprising:

a. first, second and third primers;

b. a ligating enzyme;

c. a polymerizing enzyme; and

d. at least four nucleotides.

50. The kit of Claim 49 further comprising:

e. a detectable marker attached to at least one of said primers.

51. The kit of Claim 49, further comprising:

e. a buffer suitable for the ligation and polymerization reactions.

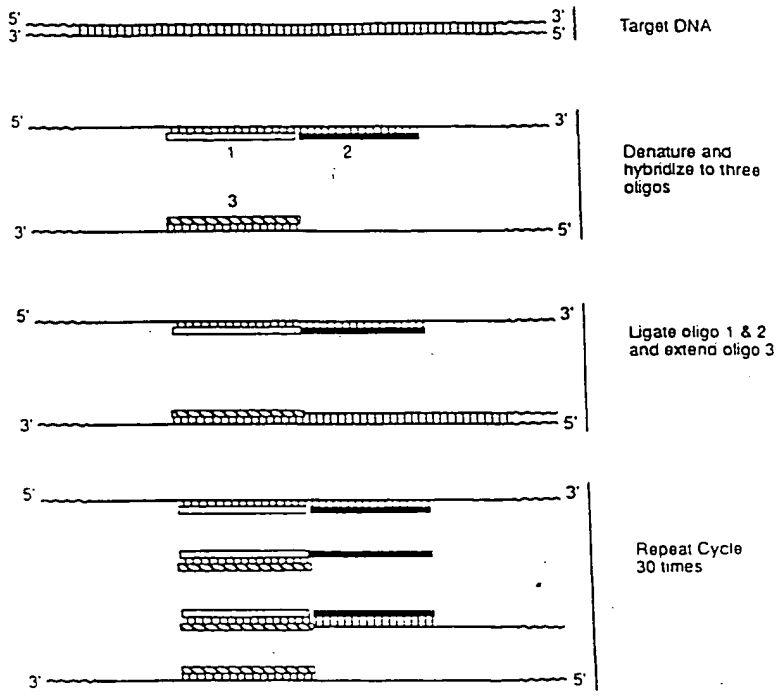
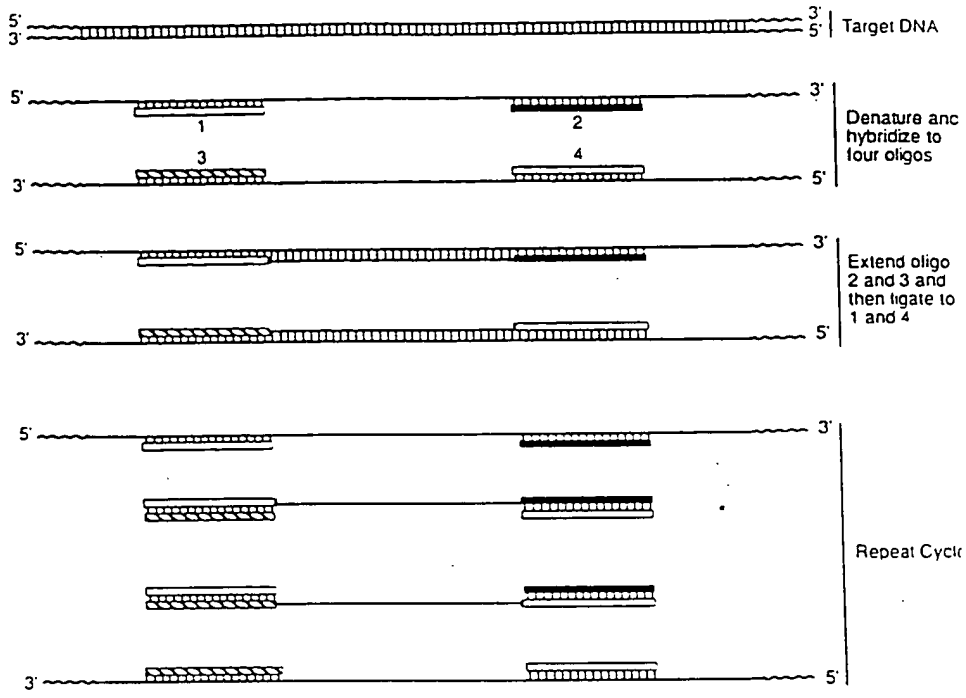

5'
3'
3'
5'
Target DNA
5'
3'
1
2
3
3'
5'
Denature and hybridize to three oligos
5'
3'
3'
5'
Ligate oligo 1 & 2 and extend oligo 3
5'
3'
3'
5'
Repeat Cycle 30 times

FIGURE 1

1 2 3 4 5 6

FIGURE 2



Modified OCR

FIGURE 3

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1921 GATTGAGAAA GCTGTCAAGG AAGCCAATGC CTATGACTTT ATCATGAAAC TGCCTCATAA
1981 ATTTGACACC CTGGTTGGAG AGAGAGGGGC CCAGTTGAGT GGTGGGCAGA AGCAGAGGAT
2041 CGCCATTGCA CGTGCCCTGG TTCGCAACCC CAAGATCCTC CTGCTGGATG AGGCCACGTC
2101 AGCCTTGGAC ACAGAAAGCG AAGCAGTGGT TCAGGTGGCT CTGGATAAGG CCAGAAAAGG
2161 TCGGACCACC ATTGTGATAG CTCATCGTTT GTCTACAGTT CGTAATGCTG ACGTCATCGC
2221 TGGTTTCGAT GATGGAGTCA TTGTGGAGAA AGGAAATCAT GATGAACTCA TGAAAGAGAA
2281 AGGCATTTAC TTCAAACTTG TCACAATGCA GACAGCAGGA AATGAAGTTG AATTAGAAAA
2341 TGCAGCTGAT GAATCCAAAA GTGAAATTGA TGCCTTGGAA ATGTCTTCAA ATGATTCAAG
2401 ATCCAGTCTA ATAAGAAAAA GATCAACTCG TAGGAGTGTC CGTGGATCAC AAGCCCAAGA
2461 CAGAAAGCTT AGTACCAAAG AGGCTCTGGA TGAAAGTATA CCTCCAGTTT CCTTTTGGAG
2521 GATTATGAAG CTAAATTTAA CTGAATGGCC TTATTTTGTT GTTGGTGTAT TTTGTGCCAT
2581 TATAAATGGA GGCCTGCAAC CAGCATTTGC AATAATATTT TCAAAGATTA TAGGGGTTTT
2641 TACAAGAATT GATGATCCTG AAACAAAACG ACAGAATAGT AACTTGTTTT CACTATTGTT
2701 TCTAGCCCTT GGAATTATTT CTTTTATTAC ATTTTTCCTT CAGGGTTTCA CATTTGGCAA
2761 AGCTGGAGAG ATCCTCACCA AGCGGCTCCG ATACATGGTT TTCCGATCCA TGCTCAGACA
2821 GGATGTGAGT TGGTTTGATG ACCCTAAAAA CACCACTGGA GCATTGACTA CCAGGCTCGC
2881 CAATGATGCT GCTCAAGTTA AAGGGGCTAT AGGTTCCAGG CTTGCTGTAA TTACCCAGAA
2941 TATAGCAAAT CTTGGGACAG GAATAATTAT ATCCTTCATC TATGGTTGGC AACTAACACT
3001 GTTACTCTTA GCAATTGTAC CCATCATTGC AATAGCAGGA GTTGTTGAAA TGAAAATGTT
3061 GTCTGGACAA GCACTGAAAG ATAAGAAAGA ACTAGAAGGT GCTGGGAAGA TCGCTACTGA
3121 AGCAATAGAA AACTTCCGAA CCGTTGTTTC TTTGACTCAG GAGCAGAAGT TTGAACATAT
3181 GTATGCTCAG AGTTTGCAGG TACCATACAG AAACTCTTTG AGGAAAGCAC ACATCTTTGG
3241 AATTACATTT TCCTTCACCC AGGCAATGAT GTATTTTTCC TATGCTGGAT GTTTCCGGTT
3301 TGGAGCCTAC TTGGTGGCAC ATAAACTCAT GAGCTTTGAG GATGTTCTGT TAGTATTTTC
3361 AGCTGTTGTC TTTGGTGCCA TGGCCGTGGG GCAAGTCAGT TCATTTGCTC CTGACTATGC
3421 CAAAGCCAAA ATATCAGCAG CCCACATCAT CATGATCATT GAAAAAACCC CTTTGATTGA
3481 CAGCTACAGC ACGGAAGGCC TAATGCCGAA CACATTGGAA GGAAATGTCA CATTTGGTGA
3541 AGTTGTATTC AACTATCCCA CCCGACCGGA CATCCCAGTG CTTCAGGGAC TGAGCCTGGA
3601 GGTGAAGAAG GGCCAGACGC TGGCTCTGGT GGGCAGCAGT GGCTGTGGGA AGAGCACAGT
3661 GGTCCAGCTC CTGGAGCGGT TCTACGACCC CTTGGCAGGG AAAGTGCTGC TTGATGGCAA
3721 AGAAATAAAG CGACTGAATG TTCAGTGGCT CCGAGCACAC CTGGGCATCG TGTCCCAGGA
3781 GCCCATCCTG TTTGACTGCA GCATTGCTGA GAACATTGCC TATGGAGACA ACAGCCGGGT
3841 GGTGTCACAG GAAGAGATCG TGAGGGCAGC AAAGGAGGCC AACATACATG CCTTCATCGA
3901 GTCACTGCCT AATAAATATA GCACTAAAGT AGGAGACAAA GGAACTCAGC TCTCTGGTGG
3961 CCAGAAACAA CGCATTGCCA TAGCTCGTGC CCTTGTTAGA CAGCCTCATA TTTTGCTTTT
4021 GGATGAAGCC ACGTCAGCTC TGGATACAGA AAGTGAAAAG GTTGTCCAAG AAGCCCTGGA
4081 CAAAGCCAGA GAAGGCCGCA CCTGCATTGT GATTGCTCAC CGCCTGTCCA CCATCCAGAA
4141 TGCAGACTTA ATAGTGGTGT TTCAGAATGG CAGAGTCAAG GAGCATGGCA CGCATCAGCA
4201 GCTGCTGGCA CAGAAAGGCA TCTATTTTTC AATGGTCAGT GTCCAGGCTG GAACAAAGCG
4261 CCAGTGAACT CTGACTGTAT GAGATGTTAA ATACTTTTTA ATATTTGTTT AGATATGACA
4321 TTTATTCAAA GTTAAAAGCA AACACTTACA GAATTATGAA GAGGTATCTG TTTAACATTT
4381 CCTCAGTCAA GTTCAGAGTC TTCAGAGACT TCGTAATTAA AGGAACAGAG TGAGAGACAT
4441 CATCAAGTGG AGAGAAATCA TAGTTTAAAC TGCATTATAA ATTTTATAAC AGAATTAAAG
4501 TAGATTTTAA AAGATAAAAT GTGTAATTTT GTTTATATTT TCCCATTTGG ACTGTAACTG
4561 ACTGCCTTGC TAAAAGATTA TAGAAGTAGC AAAAAGTATT GAAATGTTTG CATAAAGTGT
4621 CTATAATAAA ACTAAACTTT CATGTG
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FIGURE 4

(SEQ. ID NO. 1)

INTERNATIONAL SEARCH REPORT

Int. ional application No.
PCT/US93/10883

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; C12P 19/34
US CL :435/6; 435/91.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 435/91.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/01069 (SEGEV) 08 FEBRUARY 1990, see pages 5, 10-12.	1-51
Y	US, A, 4,683,195 (MULLIS ET AL.) 28 JULY 1987, see entire document.	1-51
Y	ERLICH ET AL., "POLYMERASE CHAIN REACTION" PUBLISHED 1990 by COLD SPRING HARBOR LABORATORY PRESS (COLD SPRING HARBOR, NY), pages 75-81, see entire document.	45

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 1994	MAR 17 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer Jill Warden for SCOTT HOUTTEMAN Telephone No. (703) 308-0196

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